Trees (2012) 26:121–129
DOI 10.1007/s00468-011-0663-6

ORIGINAL PAPER

Differential gene expression in leaves of a scab susceptible and a resistant apple cultivar upon *Venturia inaequalis* inoculation

Christine Holzapfel · Barbara Meisel · Fritz Thümmler · Christoph Leser · Dieter Treutter

Received: 8 March 2011 / Revised: 2 August 2011 / Accepted: 29 November 2011 / Published online: 13 December 2011

Abstract Apple scab, one of the most damaging diseases in apple worldwide is caused by the fungal pathogen *Venturia inaequalis*. Pathogen-induced gene expression was analyzed in leaves of greenhouse- and field-grown trees of the scab susceptible cultivar Golden Delicious and the resistant cultivar Rewena using the macroarray technique. The results show that the defence of the *Vf*-resistant cultivar Rewena is based on different mechanisms than the basal defence response of the susceptible Golden Delicious: whereas lignification seems to play an essential role in scab defence of Rewena, a thaumatin-like as well as a flavonoid gene are assumed to be involved in the mostly insufficient defensive response of Golden Delicious. Furthermore, a method was developed for quantification of the *V. inaequalis* in infected leaves using real-time quantitative reverse transcription-PCR.

Keywords *Malus domestica* · *Venturia inaequalis* · Differential gene expression · Lignification

Communicated by W. Osswald.

A contribution to the Special Issue: Pome Fruit Health.

C. Holzapfel · C. Leser · D. Treutter (✉)
Unit of Fruit Science, Centre of Life and Food Sciences Weihenstephan, Technische Universität München, Dürnast 2, 85354 Freising, Germany
e-mail: dieter.treutter@wzw.tum.de

B. Meisel
Monsanto South Africa (Pty) Ltd, 2 Vermeulen St, Petit 1512, South Africa

F. Thümmler
vertis Biotechnologie AG, Lise-Meitner-Str. 30, 85354 Freising, Germany

Abbreviations

APX	Ascorbate peroxidase
CAD	Cinnamyl alcohol dehydrogenase
CHS	Chalcone synthase
Ct	Threshold cycle
GD	Golden Delicious
PAL	Phenylalanine ammonia lyase
P_{Bon}	Bonferroni-corrected *P* value
Re	Rewena

Introduction

The scab disease, caused by the fungus *Venturia inaequalis*, is responsible for huge economical losses worldwide. Most of the resistant apple varieties, such as Rewena, bear the *Vf* gene from *Malus* × *floribunda* Sieb. clone 821, which is now named as *Rvi6* gene (Bus et al. 2009). This gene represents the most important resistance gene in breeding programs (Fischer and Fischer 1996; Gessler et al. 2006). However, nothing is known about the physiological and constitutive function of this resistance gene. The susceptible cultivar Golden Delicious bears the race-specific *Vg* (*Rvi1*) scab-resistance gene (Jha et al. 2009; Bowen et al. 2011). This variety shows pathogen-related responses being mostly not sufficient for defence of the fungus. The degree of its susceptibility is modifiable by environmental conditions (Mayr et al. 1997; Leser and Treutter 2005). According to the definitions described by Ingle et al. (2006), the resistant cultivar Rewena might express a cultivar-specific resistance with resistance genes inactivating the fungal effectors. Following the model of Jones and Dangl (2006) this might be regarded as effector-triggered

immunity whereas the behaviour of Golden Delicious might be classified as effector-triggered susceptibility. A few studies have already focused on the up- and down-regulation of genes or proteins in the apple–*V. inaequalis* interaction. Komjanc et al. (1999) reported a higher *V. inaequalis* induced accumulation of receptor-like protein kinase (LRPKm1) transcripts from *M. domestica* in the resistant cultivar Florina compared to the susceptible cultivar Golden Delicious. According to Poupard et al. (2003), a pathogenesis related (PR 10) gene from apple was up-regulated in Golden Delicious upon inoculation either with a virulent or an avirulent strain of *V. inaequalis*. The induction occurred earlier in the compatible interaction but at higher levels in the incompatible one. Gau et al. (2004) observed an induction of several PR proteins (β-1,3-glucanase, chitinase, thaumatin) in the apoplastic fluid of the inoculated susceptible cultivar Elstar, and the presence of these PR proteins in the resistant cultivar Remo without inoculation. Degenhardt et al. (2005) reported that the scab resistant cultivar Remo shows high constitutive expression of genes encoding PR proteins such as β-1,3-glucanase, ribonuclease-like PR 10, or cysteine protease inhibitor. They also investigated that the susceptible cultivar Elstar accumulates lower amounts of those transcripts in non-inoculated leaves but shows up-regulation upon *V. inaequalis* inoculation.

The aim of the present study was the analysis of genes differentially expressed in leaves of greenhouse- and field grown apple trees during the infection period of *V. inaequalis* in the scab susceptible cultivar Golden Delicious and the resistant cultivar Rewena.

Materials and methods

Plant material and treatment

The following apple cultivars were chosen for the experiments: Golden Delicious as a genotype susceptible to apple scab but partially showing defence reactions and resistant against race 7 of *V. inaequalis* mediated by the V_g resistance gene *Rvi1* (Bénaouf and Parisi 1997), and Rewena as a scab resistant variety containing the *Vf*-resistance gene *Rvi6* from *Malus* × *floribunda* Sieb. clone 821 (Fischer and Fischer 1996) conferring resistance to scab races 1–6 and 8 (Gessler et al. 2006; Bowen et al. 2011). For the artificial *V. inaequalis* inoculation, 3-year-old trees grafted on rootstock M9 were grown in peat substrate in a greenhouse covered with UV-permeable plastic. Conidia of *V. inaequalis* were washed from leaves of field-grown apple trees showing intense sporulating scab lesions and were then used for inoculation in a concentration of about 5×10^6 conidia/ml. The inoculation was performed by spraying the whole trees with the spore solution. In order to provide continuous leaf wetness during the inoculation period of 3 days, the trees were over-crown irrigated. In addition, non-inoculated control plants were cultured mock-sprayed with water under the same conditions. Pooled samples consisting of ten to eleven leaves from three to four trees were taken 1, 2, 4, 8, 14, and 25 dpi, frozen in liquid nitrogen, and stored at −80°C for gene expression analyses. Golden Delicious leaves from 25 dpi were additionally sorted according to their macroscopically visible infected leaf area: <30% infected leaf area: weak infection, >30% infected leaf area: strong infection. Unless otherwise noted, the leaves with highest percentage of infected leaf area were used for expression studies. At the same time, a field experiment with natural *V. inaequalis* inoculation took place using the same cultivars. Sampling of leaf position 1, 2, and 4 took place on day 1, 2, 3, 4, 7, and 8 when on about 4-week-old Golden Delicious leaves the first scab symptoms were visible indicating a hundred percent infection. Sample processing was carried out as described for the greenhouse samples.

RNA isolation and in vitro RNA synthesis

Total RNA from apple leaves was isolated following a modified protocol according to Chang et al. (1993). *V. inaequalis* cultures were grown on malt medium in Petri dishes. A colony of actively growing mycelium was cut from the plates and added to 50 ml of potato-dextrose broth in 250 ml flasks. After an incubation period of about 3 weeks in the dark at room temperature, the mycelium was frozen in liquid nitrogen, ground to a fine powder, and stored at −80°C. Fungal RNA was isolated using TRIzol® Reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions.

In vitro RNA synthesis was used in order to amplify RNA serving as a spiking control in the array hybridization experiments. The selected construct pMM14 is composed of the 3′-untranslated region of the luciferin-binding protein from the dinoflagellate *Gonyaulax polyedra* (Mittag et al. 1994), cloned in the vector pTZ18R (Pharmacia, Freiburg, Germany), and was kindly provided by Dr. M. Mittag (University Jena, Germany). An internal standard DNA stock was produced by culturing the clones containing the inserts and extracting pMM14 DNA by using the QIAfilter Plasmid Maxi Kit (Qiagen, Düsseldorf, Germany). RNA was in vitro synthesized from the T7-promoter according to the instructions of the supplier of the T7-polymerase (MBI Fermentas, St. Leon-Rot, Germany). Quality of all RNAs was verified by gel electrophoresis and quantity was measured spectrophotometrically at 260 nm.

cDNA library construction

Three cDNA libraries were constructed by vertis Biotechnologie AG, Freising, Germany, using a subtractive hybridization technique previously described by Ros et al. (2004). One library was enriched for late *V. inaequalis* induced genes in Golden Delicious (20–28 days after inoculation), another for early *V. inaequalis* induced genes in the resistant cultivar Rewena (1–7 days after inoculation), and a third one for late induced genes in Rewena (20–28 days after inoculation). In all cases, 2 µg of apple leaf RNA were used as tester or driver, respectively. *V. inaequalis* RNA was added to the driver RNA in order to eliminate pathogen-derived genes. cDNA synthesis was carried out by using M-MLV-RNase H-reverse transcriptase (MBI Fermentas). Different oligo(dT)-linker-primers were used in order to obtain the tester and the driver cDNA, respectively. Double strand synthesis of the tester and driver cDNA was carried out via Klenow DNA-polymerase using different random-adapter primers. The cDNA was amplified in a long and accurate PCR (LA-PCR) according to Barnes (1994). In a subtraction step, single stranded ss-cDNA was obtained from the tester and driver cDNA. The ss-cDNA of the tester was then hybridized with a tenfold excess of the driver ss-cDNA. The re-associated, double stranded tester/driver ds-cDNA was separated from the remaining ss-cDNA of the tester using hydroxylapatite columns. The subtractive ss-cDNA was then amplified in a further LA-PCR using tester-specific primers. In a second subtractive hybridization, S2-cDNA was obtained using S1-cDNA as tester.

The cDNA was ligated into plasmid pBluescript II SK (+) (Stratagene, Mannheim, Germany) and transformed into NovaBlue chemically component *E*scherichia *coli* cells (Invitrogen). Finally, the transformation assay was plated on LB-Ampicillin medium in Petri dishes and cultured overnight at 37°C.

PCR amplification of cDNA inserts

Bacterial colonies were randomly picked from the plated subtractive cDNA libraries and cultured overnight in 1 ml of LB medium supplemented with 2.5 µl/ml Ampicillin in 96-deepwell plates at 37°C and 300 rpm. The cDNA inserts of the clones were amplified by PCR in a Perkin-Elmer GeneAMP PCR System 9600 thermocycler (Rodgau, Germany) using the primers 5′ pBL2SK GACTGG AAAGCGGGCAGTGAG (forward) and 5′ pBL2SK GTTGAATTAGCGGAACGTCGT (reverse), flanking the cloning site of pBluescript II SK (+) (Stratagene). PCR reactions contained 27 µl of distilled water, 0.05 µl of each 100 µM primer, 3 µl 10× Advantage Buffer (Amersham Biosciences, Freiburg, Germany), 0.125 U home-made Taq (Pluthero 1993), and 1 µl bacterial culture. PCR was run at 95°C for 2 min followed by 35× (95°C for 15 s, 55°C for 30 s, 72°C for 3 min). In order to amplify a bigger amount of PCR products, additional PCR reactions were carried out with the primers 5′ pBSKoL ATGCTTCCGGCTCGT ATGTT (forward) and 5′ pBSKoR AGCACTGACCCTT TTGGGAC (reverse), using the same PCR conditions as above. Quality of all PCR products was checked by gel electrophoresis.

Sequence analysis

All amplified cDNA fragments were purified and concentrated through MultiScreen®-PCR Plates (Millipore, Schwalbach am Taunus, Germany). The ABI PRISM® BIG DYE Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, CA, USA) was used for sequence analyses. In order to generate partial 5′ sequence information of the cDNA clones, T7 primer (GTAA TACGACTCACTATAGGGC) was used. PCR reactions were run in a Perkin-Elmer GeneAMP PCR System 9600 thermocycler (Rodgau) and analyzed using an ABI PRISM® 377 DNA sequencer (Perkin-Elmer). Sequence data were analyzed with the Sequencing Analysis Software version 3.2 ABI PRISM® (Perkin-Elmer). Sequence homologies were tested at nucleotide and protein level using the NCBI BLAST server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). *E* values (expect values) greater than e-10 were considered as not statistically significant. Additionally, sequence alignment studies were carried out using the SDSC Biology WorkBench 3.2 software. Clones exhibiting identities ≥80% on nucleotide level were in the following handled as identical clones. The new sequence data are available in the GenBank under the accession numbers EG025807–EG025983.

Gene expression analysis

According to their putative function and the first hybridization results, 177 unigenes and 7 additional flavonoid clones (Fischer et al. 2006) were selected for further gene expression studies and spotted for cDNA macroarray analyses using a 96 Solid Pin Multi-Blot Replicator (V&P Scientific, San Diego, CA, USA). 1 µl of each purified and concentrated PCR product was applied in duplicate to positively charged nylon membranes (Pall Biodyne B, Nunc, Roskilde, Denmark). pMM14 was spotted on each membrane as spiking control. The membranes were directly washed in 0.2 M NaOH, 0.2 M SDS at 65°C for 20 min, followed by a 15 min wash in 30 mM Tris pH 7.5 at room temperature. First-strand cDNA synthesis and simultaneous labelling with α-^{32}P-labelled nucleotides

(Hartmann Analytic, Braunschweig, Germany) were performed on total RNA using oligo-dT primers. The 13 µl reactions in DEPC-treated water containing 10 µg of total RNA, 40 pg of spiking RNA prepared from pMM14 and 2 µg of $(dT)_{12\text{-}18}$ primer (Roche, Basel, Switzerland) were denatured at 72°C for 2 min and immediately cooled down to 42°C. Reverse transcription was performed in a total volume of 22.5 µl using REVERTAID M-MuLV reverse transcriptase (MBI Fermentas). The samples were incubated for 1 h at 42°C together with 0.33 mM each of dATP, dGTP, and dTTP, 3.3 µM dCTP, and 10 µCi of [α-^{32}P]dCTP (3,000 Ci/mmol, Hartmann Analytic). The reaction was stopped by adding 150 mM NaOH and 10 mM EDTA, and then RNA was hydrolyzed for 30 min at 65°C. In order to remove the unincorporated nucleotides, the reaction mixture was gel-filtrated through SEPHADEX G-50 columns (Amersham Biosciences). The incorporation of [α-^{32}P]dCTP was checked by scintillation counting. Afterwards, the samples were denatured at 95°C for 5 min. The membranes were prehybridized for 2 h in Church buffer (Church and Gilbert 1984), containing 0.25 M $NaPO_4$ pH 7.2, 7% SDS, 1 mM EDTA, 250 µg tRNA (Roche), and 2.5 µg oligo $d(A)_{40\text{–}60}$ (Amersham Biosciences). Hybridization took place in the same buffer at 65°C overnight. Afterwards, the arrays were washed twice in 2× SSC, 0.1% SDS and twice in 40 mM $NaPO_4$ pH 7.2, 0.1% SDS at 65°C, for 20 min each. Finally, the membranes were exposed to imaging screens (Kodak, Stuttgart, Germany) for about 24 h.

Gene expression data analysis

Hybridization signals were detected using STORM 860 (Molecular Dynamics, Freiburg, Germany) with a resolution of 50 µm. The image data obtained were analyzed with the program package Array Vision 8.0 (Imaging Research, Munich, Germany) which calculated normalized volumes (nVols) for each spot. In order to have equal gene expression levels in all experiments, the nVol of each spot was divided by the mean value of pMM14 serving as a reference. For each sample, the nVols of at least two independent hybridization experiments were considered.

Real-time quantitative reverse transcription-PCR (qRT-PCR)

For quantification of the input amount of target RNA from *V. inaequalis* in apple leaves, primers were designed for a *V. inaequalis*-derived gene showing highest homology to a hypothetical gene MG08918.4 from *Magnaporthe grisea*, the causal agent of the rice blast disease. This gene information derived from a cDNA library enriched for *V. inaequalis* induced genes also containing *V. inaequalis* genes itself. An absolute quantification was carried out using the standard curve method according to the following formula:

$$\text{Gene expression} = \frac{C_{t_{\text{y axis intercept}}}}{\text{slope}}$$

The C_t (threshold cycle) value is defined as that cycle number at which a statistically significant increase in the fluorescence can first be detected.

Freshly prepared dilutions from RNA from the in vitro grown pathogen were therefore used as standard templates. Additionally, gel electrophoresis confirmed that the signal was the result of product amplification.

Statistical analysis

Statistical analysis of the gene expression results was conducted with SAS, version 9.1 (SAS Institute Inc. 2003, Munich, Germany) according to established methods used for microarray analyses (Quackenbush 2001; Cui and Churchill 2003). The $\log_2$-transformed data were analyzed using two interconnected mixed models (PROC MIXED) according to Wolfinger et al. (2001). In the first model, the log-transformed raw data were normalized. In the second model, the normalized residues of the first model were used for each gene considering fixed (cultivar, leaf age, and *V. inaequalis* and mock-inoculation) and random factors (array). All parameters were fitted simultaneously into the model by REML-estimation (restricted maximum likelihood). Test-hypotheses of interest were constructed using ESTIMATE-statements. In order to identify significant differences between data sets, mixed model-based *t* tests were carried out. *P* values were Bonferroni-corrected (P_{Bon}) in order to control the multiplicity problem occurring when analyzing numerous genes simultaneously. In case of high differences between two compared conditions P_{Bon} values, in case of lower differences conventional *P* values were considered. Such data analysis assured not to neglect significantly differentially expressed genes with low fold-expression differences.

Results

Identification and quantification of the *V. inaequalis* infection

The artificial *V. inaequalis* inoculation in the greenhouse led to relatively weak scab symptoms with maximal 45% disease index. The first symptoms of apple scab on inoculated leaves of the susceptible cultivar Golden Delicious occurred about 3 weeks after inoculation. No symptoms were visible on non-inoculated Golden Delicious leaves

and on leaves of the resistant variety Rewena. In addition to this visible evaluation, real-time detection with sequence specific primers for a *V. inaequalis*-derived gene showing highest homology to a clone from the fungus *M. grisea* was performed in order to quantify the inoculation. Linear regression analysis between the C_t and $\log_{10}$ of serial dilutions of *V. inaequalis* RNA was performed. The regression line fit the data to a high degree ($R^2 = 0.96$; $P \leq 0.01$; $y = -4.17x + 23.09$). According to the standard curve, the amount of *V. inaequalis* cDNA per 250 ng of total RNA from apple leaves was calculated for each sample. The qRT-PCR data corresponded very well to the number of visible lesions. In greenhouse samples of the susceptible cultivar, *V. inaequalis* cDNA was not detected in high amounts until 25 dpi, whereas no significant signal was measured in non-inoculated leaves and in the resistant variety Rewena.

In the field experiment, young Golden Delicious leaves did not yet show any scab symptoms before an incubation period of 4 weeks. Thereafter they exhibited a 100% infection, the presence of *V. inaequalis* cDNA in apple leaves ahead of the first macroscopically visible symptoms was proved by qRT-PCR analyses and values were in a range 5–20 ng/250 ng apple RNA. As in the greenhouse, no significant amounts of pathogen cDNA were found in leaves of the resistant variety Rewena.

Sequence analyses

Gene expression profiling was carried out using clones from three different subtractive cDNA libraries. 1,152 cDNA clones were randomly picked from the cDNA libraries and partially sequenced. After BLASTn and BLASTx searches (Altschul et al. 1990) and sequence alignments, 815 individual genes remained. Around 100 of the redundant clones encoded a metallothionein-like protein AMT2 from *M. domestica*, among them 80 clones in the library enriched for ontogenetically induced genes in Rewena. Overall, 43% of all sequences showed no homology to previously published sequences, and 26% showed homology to a sequence with unknown function. In all cDNA-libraries, clones encoding flavonoid genes, PR genes, and other stress- and/or defence-related genes were found. According to their putative biological function and to first gene expression results 177 clones were selected for further expression analyses. Table 1 gives information on the functional classification of the unigenes.

Gene expression analyses

Gene expression differences in the leaves of the cultivars Golden Delicious and Rewena both non-inoculated and inoculated were analyzed. In order to identify the genes involved in the apple–*V. inaequalis* interaction, especially those genes with significant differential expression in both greenhouse and field experiment were considered. The volcano plots in Fig. 1 give an overview of the significance and expression ratios of the analyzed clones. Differences in gene expression between cultivars were higher in the field experiment, those between strongly and weakly infected leaves were much higher than differences between inoculated and non-inoculated ones.

Table 1 Classification of the unigenes selected for gene expression analyses

Class	%
Stress/defence	33.3
Cellular metabolism	39.6
Transcription/translation	7.9
Photosynthesis/chloroplast	8.5
Growth	7.9
Phytohormone-related	1.1
Pathogen-derived	1.7

Comparison between inoculated and non-inoculated leaves of Golden Delicious

Differences between inoculated and non-inoculated leaves were only studied in the greenhouse since under these conditions the infection period was well defined. There was no significant effect with $P_{Bon} < 0.1$. On this stringent significance level also no cultivar-specific differences were observed. In the following, significantly differentially expressed genes with $P < 0.05$ are therefore taken into account. Highest fold change in Golden Delicious was observed for a clone with homology to a heat shock protein HSP82 (hsp82) gene from *Zea mays* with 1.6-fold higher expression in inoculated compared to non-inoculated leaves. In the resistant cultivar Rewena, a clone with homology to MdSPDS2a mRNA for spermidine synthase from *M. domestica* was 1.4-fold up-regulated upon *V. inaequalis* inoculation.

In order to learn more about the stress reaction of the susceptible cultivar, it was sought for genes being differentially expressed between non-inoculated and inoculated Golden Delicious leaves on the one hand and between weakly and strongly infected Golden Delicious leaves 25 dpi on the other hand. Out of 114 genes differentially expressed with $P < 0.05$ in the two experiments, only four clones showed significant differences in both experiments (Table 2). Except one clone with highest homology for a major allergen and lipid transfer protein Mal d 3 mRNA, the other genes were up-regulated upon inoculation or strong infection, respectively.

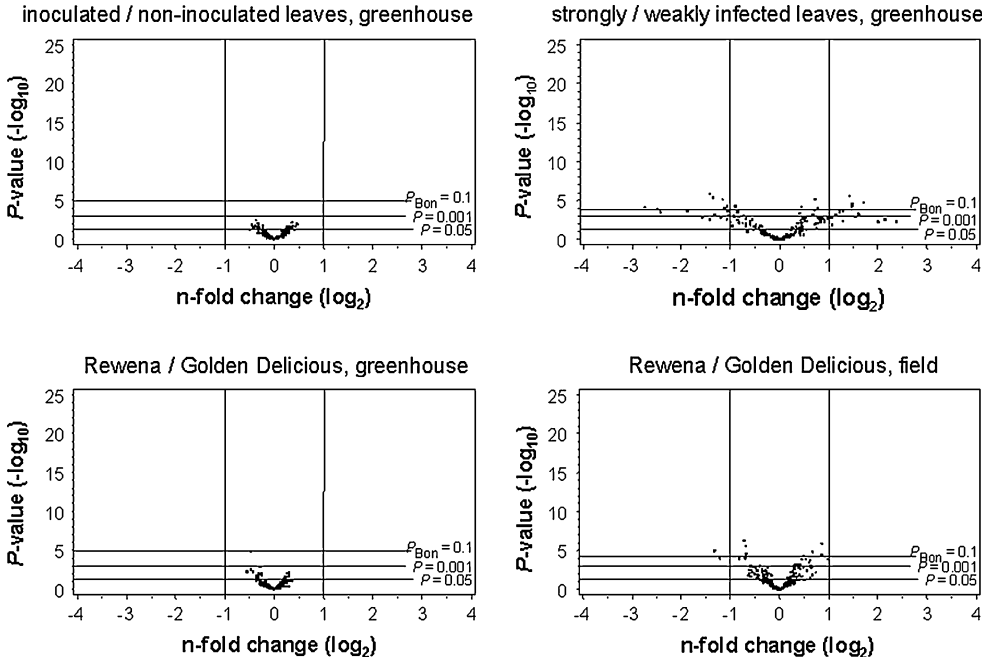

Fig. 1 *Volcano plots* with negative $\log_{10}$-transformed *P* values of the ANOVA significance test on the *y* axis and the according $\log_2$-transformed gene inductions on the *x* axis. *Dots above a horizontal line* significant on a certain level of significance. Values beyond the two *vertical lines* $\log_2$-transformed gene induction/repression ≥ 1.0

Table 2 Differences in gene expression ($P < 0.05$) occurring between both non-inoculated and inoculated Golden Delicious leaves, and between weakly and strongly infected Golden Delicious leaves 25 dpi

GenBank acc. no.	Homology	Inoculated/non-inoculated		Strongly/weakly infected	
		Ratio	*P*	Ratio	*P*
EG025920	AJ243427 mRNA for thaumatin-like protein (tl gene), *Malus domestica*	1.5	0.0370	1.6	0.0020
EG025981	X70981 CYP71A1 mRNA for P450 hydroxylase, *Solanum melongena*	1.4	0.0252	2.0	0.0184
EG025853	AAF08296 non-reversible glyceraldehyde-3-phosphate dehydrogenase, *Apium graveolens*	1.3	0.0058	2.2	<0.0001
EG025967	AY792997 major allergen and lipid transfer protein Mal d 3 mRNA, *Malus domestica*	0.7	0.0048	0.4	0.0039

Comparison between Golden Delicious and Rewena

With a significance level of $P_{Bon} < 0.05$ transcript levels from 19 genes showed cultivar dependent differences in the greenhouse experiment, and from 80 genes in the field experiment. Among these genes, 13 were found to be significantly differentially expressed in both environments (Table 3). Among the seven genes being higher expressed in Rewena, four show homology to clones related to primary metabolism.

Discussion

Macroscopically visible scab symptoms correlated largely with the concentrations of *V. inaequalis* cDNA in the apple leaves detected by qRT-PCR analyses. In the greenhouse samples, significant amounts of pathogenic cDNA were not detected before 25 dpi providing evidence of the slow fungal growth in apple leaves. In the field samples, *V. inaequalis* cDNA was detected ahead of the first scab symptoms suggesting that the intensity of infection was higher compared to the artificial inoculation under greenhouse conditions.

The major aims of these studies were to identify transcripts of apple being related to response of the resistant apple cultivar Rewena against the fungal pathogen *V. inaequalis* and of genes being involved in the stress and/or basal defence response of the susceptible cultivar Golden Delicious upon inoculation. In order to identify such clones, transcript analyses were carried out with Rewena and Golden Delicious leaves. In terms of repeatability, artificially infected leaves from the greenhouse as well as naturally infected leaves from the orchard were analysed.

Table 3 Significant differences in gene expression ($P < 0.05$) between the cultivars Golden Delicious and Rewena occurring in both greenhouse and field experiment

GenBank acc. no.	Homology	Re/GD greenhouse		Re/GD field	
		Ratio	*P*	Ratio	*P*
EG025870	AB043961 mRNA for oxygen evolving enhancer protein 2, *Bruguiera gymnorhiza*	1.3	0.0350	1.5	0.0033
EG025828	X14609 mRNA for NAPH-dependent hydroxypyruvate reductase (EC 1.1.1.29), *Cucumis sativus*	1.2	0.0097	1.6	<0.0001
EG025888	AB032245 psbP mRNA for 23 kDa polypeptide of the oxygen-evolving complex of photosystem II, *Cucumis sativus*	1.3	0.0344	1.6	<0.0001
EG025914	AF159630 cytosolic ascorbate peroxidase (APX) mRNA, APX42 allele, *Fragaria ananassa*	1.4	0.0024	1.2	0.0211
EG025935	AF053084 putative cinnamyl alcohol dehydrogenase (CAD) mRNA, *Malus domestica*	1.2	0.0011	1.2	0.0016
EG025960	AY220078 photosystem I reaction centre subunit X psaK mRNA, nuclear gene for chloroplast product, *Nicotiana tabacum*	1.4	<0.0001	1.6	<0.0001
EG025831	AAR92154 putative cysteine protease 1, *Iris hollandica*	1.3	0.0046	1.3	0.0022
EG025861	CAA52903 annexin, *Medicago sativa*	0.9	0.0411	0.8	0.0251
EG025834	AF336307 auxin-repressed protein like-protein mRNA, *Malus domestica*	0.8	0.0081	0.8	0.0006
EG025859	AY347864 clone 12-1 small Ras-like GTP-binding protein, *Malus domestica*	0.9	0.0206	0.8	0.0026
EG025941	AY347838 clone 19-6 cysteine proteinase mRNA, *Malus domestica*	0.8	0.0070	0.7	0.0015
EG025981	X70981 CYP71A1 mRNA for P450 hydroxylase, *Solanum melongena*	0.8	0.0239	0.7	0.0003
EG025957	AB072916 MdSPDS2a mRNA for spermidine synthase, *Malus domestica*	0.8	0.0102	0.7	0.0326

Among seven genes significantly higher expressed in Rewena than in Golden Delicious four clones showed highest homology to genes involved in primary metabolism. One clone possibly related to lignification was significantly higher expressed in Rewena compared to Golden Delicious, namely a putative cinnamyl alcohol dehydrogenase (CAD) mRNA. Beside that a cytosolic ascorbate peroxidase (APX) mRNA was constitutively higher expressed in Rewena. APX is an H_2O_2-detoxifying enzyme rapidly induced in response to various stresses that result in an accumulation of reactive oxygen species (ROS) (Mittler et al. 1999). Faize et al. (2004) observed an induced APX-activity upon *V. nashicola* inoculation of a scab resistant pear cultivar. As peroxidases also are involved in lignin biosynthesis, the authors suggest that lignification plays an important role in this kind of resistance. Such lignin forming peroxidases build the PR-9 protein family. CAD catalyzes the NADPH-dependent reduction of cinnamaldehydes to the corresponding alcohols and is considered a key enzyme in lignin biosynthesis (Kutsuki et al. 1982). Lignification plays an important role in defence against biotic and abiotic stresses. An accumulation of lignin often occurs in cells around an infection (Vance et al. 1980) due to an activation of the phenylpropanoid metabolism (Hahlbrock and Scheel 1989). This so-called defence lignin (Hawkins and Boudet 2003) protects healthy tissue from water losses and infections. Increased CAD-enzyme activities or transcript levels upon elicitation by fungal pathogens, wounding, or ozone have been observed, for example, by Grand et al. (1987), Galliano et al. (1993), Mitchell et al. (1994), and Coelho et al. (2006). Valentines et al. (2005) observed a very good correlation between lignin content and *Penicillium expansum*-resistance in apple and Nafussi et al. (2001) between lignification and *P. digitatum*-resistance in citrus.

A transcript significantly higher expressed in the scab susceptible cultivar Golden Delicious compared to Rewena exhibited homology to a flavonoid 3′5′-hydroxylase (F3′5′H) gene from *Verbena hybrida*. F3′5′H is a cytochrom P450-enzyme converting naringenin and eriodictyol to pentahydroxyflavanone as well as dihydrokaempferol and dihydroquercetin to dihydromyricetin (Holton et al. 1993; Seitz et al. 2006). In apple leaves, the concerned enzyme is assumed to exhibit only F3′H-activity, catalyzing the hydroxylation of naringenin to eriodictyol providing precursors of the flavan-3-ol pathway.

The comparison of non-inoculated and inoculated Golden Delicious leaves on the one hand and weakly and strongly infected Golden Delicious leaves on the other hand provided evidence of *V. inaequalis* induced genes in the susceptible cultivar. These genes may represent the basal defence response which is strongly inhibited or blocked by the pathogen. The inoculation or strong infection, respectively, led to an enhanced expression of a clone with homology to a thaumatin-like gene. Corresponding to this, Gau et al. (2004) observed a *V. inaequalis* induced up-regulation of a thaumatin-like protein in the intercellular washing fluid of the scab susceptible cultivar Elstar.

Thaumatins have the ability of degrading fungal cell walls (Stintzi et al. 1993) what may explain the high expression upon infection. Another PR gene was significantly down-regulated upon infection: A clone with homology to a major allergen and lipid transfer protein *Mal d 3* gene from *M. domestica* was significantly repressed which is attributed to very high constitutive expressions in healthy leaves. Possibly, the high constitutive gene expression level is involved in general stress and/or defence reactions. This gene repression goes in line with former results of Gau et al. (2004) who found a lipid transfer protein to decline during the first week after *V. inaequalis* inoculation in the apoplast of the scab susceptible apple cultivar Elstar. The above mentioned P450 hydroxylase clone probably involved in flavonoid biosynthesis was significantly higher expressed in inoculated and strongly infected leaves compared to non-inoculated and weakly infected leaves. This again suggests an involvement of this clone in stress and/or defence reactions of the susceptible cultivar Golden Delicious upon *V. inaequalis* inoculation. Altogether, gene expression differences between weakly and strongly infected leaves turned out to be much higher than differences between non-inoculated and inoculated leaves. This might be due to different experimental setups: while for the analyses of non-inoculated and inoculated leaves samples from 1 to 27 days old leaves were considered, the differentially infected leaves were 25–27 days old.

According to the gene expression results, we rather suggest that lignification plays a role in the defence reaction of the scab resistant cultivar Rewena against *V. inaequalis*. The formerly observed loss of resistance in another scab resistant cultivar (Mayr et al. 1997) by a general blockage of the phenylpropanoid biosynthesis through inhibition of PAL may now be explained as an inhibition of lignification. In contrast, the susceptible cultivar Golden Delicious expressed a thaumatin-like clone upon *V. inaequalis* infection which goes in line with the results on the protein level observed by Gau et al. (2004). In addition, the pathogen-induced up-regulation of a *F3'5'H* gene which is assumed to be responsible for 3′-hydroxylation of flavonoids in Golden Delicious fits in the accumulation of catechins often observed in infected apple leaves (Mayr et al. 1997; Leser and Treutter 2005; Treutter and Feucht 1990; Picinelli et al. 1995; Rühmann et al. 2002). These results give new insights into the response of apples, which are resistant or susceptible to the scab fungus and may help to understand mechanisms of basal defence and cultivar-specific resistance.

Acknowledgments This work is part of 'Sonderforschungsbereich 607' funded by the German Research Foundation (DFG). We thank A. Forstner for excellent technical assistance and the COST action 864 for providing an international frame of expertise.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic logical alignment sequence technique. J Mol Biol 215:403–410

Barnes WM (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proc Natl Acad Sci USA 91:2216–2220

Bénaouf G, Parisi L (1997) Pathogenicity of *Venturia inaequalis* strains from *Malus floribunda* 821: comparison with race 6 on apple clones. In: Berrie AM, Xu XM, Harries DC, Roberts AL, Evans K, Barbara DJ, Gessler C (eds) Integrated control of pome fruit diseases.IOBC/WPRS Bull 20, Croydon, pp 8–11

Bowen JK, Mesarich CH, Bus VG, Beresford RM, Plummer KM, Templeton MD (2011) Venturia inaequalis: the causal agent of apple scab. Mol Plant Pathol 12:105–122

Bus V, Rikkerink E, Aldwinckle HS, Caffier V, Durel CE, Gardiner S, Gessler C, Groenwold R, Laurens F, leCam B, Luby J, Meulenbroek B, Kellerhals M, Parisi L, Patocchi A, Plummer K, Schouten HJ, Tartarini S, van de Weg WE (2009) A proposal for the nomenclature of *Venturia inaequalis* races. Acta Hortic 814:739–746

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Coelho AC, Horta M, Neves D, Cravador A (2006) Involvement of a cinnamyl alcohol dehydrogenase of *Quercus suber* in the defence response to *Phytophthora cinnamomi*. Physiol Mol Plant Pathol 69:62–72

Cui X, Churchill GA (2003) Statistical tests for differential expression in cDNA microarray experiments. Genome Biol 4(210):1–10

Degenhardt J, Al-Masri AN, Kurkcuoglu S, Szankowski I, Gau AE (2005) Characterization by suppression subtractive hybridization of transcripts that are differentially expressed in leaves of apple scab-resistant and susceptible cultivars of *Malus domestica*. Mol Genet Genomics 273:326–335

Faize M, Faize L, Ishizaka M, Ishii H (2004) Expression of potential defense responses of Asian and European pears to infection with *Venturia nashicola*. Physiol Mol Plant Pathol 64:319–330

Fischer C, Fischer M (1996) Results in apple breeding at Dresden-Pillnitz—a review. Gartenbauwissenschaft 61:139–146

Fischer TC, Halbwirth H, Roemmelt S, Sabatini E, Schlangen K, Andreotti C, Spinelli F, Costa G, Forkmann G, Treutter D, Stich K (2006) Induction of polyphenol gene expression in apple (*Malus* × *domestica*) after the application of a dioxygenase enzyme inhibitor. Physiol Plant 128:604–617

Galliano H, Cabané M, Eckerskorn C, Lottspeich F, Sandermann H, Ernst D (1993) Molecular cloning, sequence analysis and elicitor-/ozone-induced accumulation of cinnamyl alcohol dehydrogenase from Norway spruce (*Picea abies* L.). Plant Mol Biol 23:145–156

Gau AE, Koutb M, Pitrowski M, Kloppstech K (2004) Accumulation of pathogenesis-related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo. Europ J Plant Pathol 110:703–711

Gessler C, Patocchi A, Sansavini S, Tartarini S, Gianfrancesci L (2006) Venturia inaequalis resistance in apple. Crit Rev Plant Sci 25:473–503

Grand C, Sarni F, Lamb C (1987) Rapid induction by fungal elicitor of the synthesis of cinnamyl alcohol dehydrogenase, a specific enzyme of lignin synthesis. Eur J Biochem 169:73–77

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Ann Rev Plant Physiol 40:347–369

Hawkins S, Boudet A (2003) 'Defense lignin' and hydroxycinnamyl alcohol dehydrogenase activities in wounded *Eucalyptus gunnii*. Forest Pathol 33:91–104

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu CY, Farcy E, Stevenson TW, Cornish EC (1993) Cloning and expression of P450 genes controlling flower colour. Nature 366:276–279

Ingle RA, Carstens M, Denby KJ (2006) PAMP recognition and the plant–pathogen arms race. BioEssays 28:880–889

Jha G, Thakur K, Thakur P (2009) The Venturia apple pathosystem: pathogenicity mechanisms and plant defense responses. J Biomed Biotechnol 2009:1–10

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Komjanc M, Festi S, Rizzotti L, Cattivelli L, Cervone F, De Lorenzo G (1999) A leucine-rich repeat receptor-like protein kinase (LRPKm1) gene is induced in *Malus* × *domestica* by *Venturia inaequalis* infection and salicylic acid treatment. Plant Mol Biol 40:945–957

Kutsuki H, Shimada M, Higuchi T (1982) Regulatory role of cinnamyl alcohol dehydrogenase in the formation of guaiacyl and syringyl lignins. Phytochemistry 21:19–23

Leser C, Treutter D (2005) Effects of nitrogen supply on growth, contents of phenolic compounds and pathogen (scab) resistance of apple trees. Physiol Plant 123:49–56

Mayr U, Michalek S, Treutter D, Feucht W (1997) Phenolic compounds of apple and their relationship to scab resistance. J Phytopathol 145:69–75

Mitchell HJ, Hall JL, Barber MS (1994) Elicitor-induced cinnamyl alcohol dehydrogenase activity in lignifying wheat (*Triticum aestivum* L.) leaves. Plant Physiol 104:551–556

Mittag M, Lee D-H, Hastings JW (1994) Circadian expression of the luciferin-binding protein correlates with the binding of a protein to the 3′ untranslated region of its mRNA. Proc Natl Acad Sci USA 91:5257–5261

Mittler R, Lam E, Shulaev V, Cohen M (1999) Signals controlling the expression of cytosolic ascorbate peroxidase during pathogen-induced programmed cell death in tobacco. Plant Mol Biol 39:1025–1035

Nafussi B, Ben-Yehoshua S, Rodov V, Peretz J, Ozer BK, D'hallewin G (2001) Mode of action of hot-water dip in reducing decay of lemon fruit. J Sci Food Chem 49:107–113

Picinelli A, Dapena E, Mangas JJ (1995) Polyphenolic pattern in apple tree leaves in relation to scab resistance. A preliminary study. J Agric Food Chem 43:2273–2278

Pluthero FG (1993) Rapid purification of high-activity Taq DNA polymerase. Nucl Acids Res 21:4850–4851

Poupard P, Parisi L, Campion C, Ziadi S, Simoneau P (2003) A wound- and etephon-inducible PR-10 gene subclass from apple is differentially expressed during infection with a compatible and an incompatible race of *Venturia inaequalis*. Physiol Mol Plant Pathol 62:3–12

Quackenbush J (2001) Computational analysis of microarray data. Nat Rev Genet 2:418–427

Ros B, Thuemmler F, Wenzel G (2004) Analysis of differentially expressed genes in a susceptible and moderately resistant potato cultivar upon *Phytophthora infestans* infection. Mol Plant Pathol 5:191–201

Rühmann S, Leser C, Bannert M, Treutter D (2002) Relationship between growth, secondary metabolism, and resistance of apple. Plant Biol 4:137–143

Seitz C, Eder C, Deiml B, Kellner S, Martens S, Forkmann G (2006) Cloning, functional identification and sequence analysis of flavonoid 3'-hydroxylase and flavonoid 3′5′-hydroxylase cDNAs reveals independent evolution of flavonoid 3′5′-hydroxylase in the *Asteraceae* family. Plant Mol Biol 61:365–381

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmanna S, Geoffroy P, Legranda M, Fritig B (1993) Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie 75:687–706

Treutter D, Feucht W (1990) The pattern of flavan-3-ols in relation to scab resistance of apple cultivars. J Hortic Sci 65:511–517

Valentines MC, Vilaplana R, Torres R, Usall J, Larrigaudière C (2005) Specific roles of enzymatic browning and lignification in apple disease resistance. Postharvest Biol Technol 36:227–234

Vance CP, Kirk TK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. Ann Rev Phytopathol 18:259–288

Wolfinger RD, Gibson G, Wolfinger E, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS (2001) Assessing gene significance from cDNA microarray expression data via mixed models. J Comput Biol 8:625–637